Prokaryotic Expression of a Novel Therapeutic recombinant transbody H2L2 Targeting Hepatitis B Virus core protein

Yanqing Li[1], Juanjuan Gao[1], Bo Wang[1], Fang Wang[1], Zhonglin Wang[1], Bing Liu[1], Lingyun Hui[1], Yiping Li[2], and Yawen Wang[1]

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Abstract

Background and Purpose Chronic Hepatitis B virus (CHB) infection is a global burden for public health. Treatment methods such as interferon and nucleoside analogues may effectively control viral infection but cannot eliminate the virus due to their inability to remove viral covalently closed circular DNA (cccDNA) inside host nuclear. The persistence of cccDNA in the infected hepatocytes is a crucial obstacle to antiviral therapies. For years, efforts had been undertaken to understand the formation and regulation of HBV cccDNA. Natural core antibodies were modified to target intracellular core proteins across the membrane to achieve antiviral effect in several studies. However, modified natural antibody had limits: 1) it was unstable inside cells; 2) it had strong immunogenicity; 3) lack of targeting. Experimental approach and Key Results Therefore, we altered natural antibody into a micro-transbody which has correct intracellular folding, stronger affinity, and weaker immunogenicity to establish a new recombinant transbody for the specific and efficient immune clearance of viruses in hepatocytes. Immunofluorescence and immunohistochemistry were used to verify the transmembrane and antigen-binding ability of our recombinant transbody. Conclusions & Implications Taken together, we demonstrate a recombinant transbody H2L2 with transmembrane and antigen-binding activity expressed by E.coli in this study, which may be an encouraging exploration of therapeutic strategies against hepatitis B infection inside hepatocellular.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contribution statement:

The main idea of this study was from Yawen Wang and Yiping Li. Yanqing Li, Juanjuan Gao and Zhonglin Wang conducted the experiments. Bo Wang and Fang Wang provided technical guidance. Yanqing Li, Juanjuan Gao, Zhonglin Wang, Bo Wang, Fang Wang and Bing Liu conducted the data analysis. Yanqing Li, Juanjuan Gao, Yawen Wang and Yiping Li wrote the first draft of the manuscript. Yawen Wang and Yiping Li revised the content of the paper. All authors contributed to and approved the final version of the manuscript.

Conflict of interest statement

The authors have no conflicts of interest to declare in relation to this work.

Consent to publication

All authors reviewed the draft and approved the submission of the manuscript.

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Summary

What is already known:

- The development of new hepatitis B virus (HBV) drugs appears to be stalling. Targeting virus inside hepatocellular.

What this study adds:

- Our recombinant transbody H2L2 with transmembrane and antigen-binding activity may be an encouraging exploration of therapeutic strategies against hepatitis B infection.

Clinical significance:

- Recombinant transbody H2L2 may be an innovative therapeutic strategy for the management of HBV infection.

Abstract

Background and Purpose

Chronic Hepatitis B virus (CHB) infection is a global burden for public health. Treatment methods

such as interferon and nucleoside analogues may effectively control viral infection but cannot eliminate the virus due to their inability to remove viral covalently closed circular DNA (cccDNA) inside host nuclear. The persistence of cccDNA in the infected hepatocytes is a crucial obstacle to antiviral therapies. For years, efforts had been undertaken to understand the formation and regulation of HBV cccDNA. Natural core antibodies were modified to target intracellular core proteins across the membrane to achieve antiviral effect in several studies. However, modified natural antibody had limits: 1) it was unstable inside cells; 2) it had strong immunogenicity; 3) lack of targeting.

Experimental approach and Key Results

Therefore, we altered natural antibody into a micro-transbody which has correct intracellular folding, stronger affinity, and weaker immunogenicity to establish a new recombinant transbody for the specific and efficient immune clearance of viruses in hepatocytes. Immunofluorescence and immunohistochemistry were used to verify the transmembrane and antigen-binding ability of our recombinant transbody.

Conclusions & Implications

Taken together, we demonstrate a recombinant transbody H2L2 with transmembrane and antigen-binding activity expressed by *E.coli* in this study, which may be an encouraging exploration of therapeutic strategies against hepatitis B infection inside hepatocellular.

Keywords: CHB; therapeutic strategies; prokaryotic expression; recombinant protein; transbody

Introduction

Hepatitis B virus (HBV) infection remains a significant burden for global public health, with over 250 million people chronically infected(Pawlotsky, 2023). Infected individuals are at high risk of developing end-stage liver diseases like cirrhosis and hepatocellular carcinoma(Sonneveld, 2020). The overall goal of chronic hepatitis B (CHB) infection treatment is to eliminate or minimize HBV virus replication. Only two classes of drugs were approved for CHB treatment, including nucleos(t)ide reverse transcriptase inhibitors, also known as nucleos(t)ide analogues, and interferon-alpha, both of which could limit HBV replication but have intolerable side effects and, most importantly, have no influence on the clearance of HBV covalently closed circular DNA (cccDNA)(Buti et al., 2016; Ghany, 2017).

Each stage of HBV life circle can potentially be a therapeutic target for the prevention of HBV infection. However, the clearance of cccDNA in the infected hepatocytes is still the key obstacle to antiviral therapies(Xia & Guo, 2020). For years, efforts have been devoted to figure out the formation and regulation of cccDNA to adopt potential therapeutic strategies targeting cccDNA. Novel HBV entry inhibitors such as myrcludex B who was currently under investigating that can prevent HBV binding to its receptors to inhibit infection (Yan et al., 2012). Since HBV capsid protein (Cp) plays a vital role in genetic material encapsidation, targeting Cp can affect several processes of the viral replication cycle(Zlotnick et al., 2015). Several capsid allosteric modulators (CAMs) such as low doses of Heteroaryldihydropyrimidines (HAPs)(Stray et al., 2005), Phenylpropenamides (PPAs, ie, AT130)(Katen, Chirapu, Finn, & Zlotnick, 2010), and the sulfamoyl benzamides (SBA) had shown effects in short-term treatment (Campagna et al., 2013). It has also been suggested that apolipoprotein B mRNA editing-enzyme catalytic polypeptide-like (APOBEC) upregulation by both IFN and lymphotoxin beta receptor (LTβR) is responsible for degradation of cccDNA(Lucifora et al., 2014). In addition, genome editing like CRISPR-Cas9 system could target the HBV genome(Bloom, Maepa, Ely, & Arbuthnot, 2018; Lin et al., 2014), and cccDNA epigenetics, such as DDB1 and SMC5/6, can inhibit HBV replication by blocking HBx(Livingston, Ramakrishnan, Strubin, Fletcher, & Beran, 2017; Minor & Slagle, 2014).Efforts had been made, still there are no completely ideal therapeutic strategies targeting cccDNA.

Our previous research had successfully constructed two transbodies HBcMAb-TAT/DHBcMAb-TAT consisting of natural HBc/DHBc antibody and TAT Protein transduction domains (PTDs) targeting on HBc and duck HBc, respectively. Their ability to cross the cell membrane and antiviral effect had been confirmed, and most importantly, these transbodies could effectively reduce the level of cccDNA(Li et al., 2017; Y. Wang et al., 2015). However, there are few limitations about transbody constructed by natural antibody：1) natural antibody has strong immunogenicity due to its molecular size；2) the disulfide bond in antibody structure is unstable under the condition of intracellular reduction, which may decrease the therapeutic effect of the antibody inside the cell；3) production of monoclonal antibodies is complex and expensive; 4) frequent using of murine monoclonal antibody can easily produce human anti-mouse antibody and the allergic reaction would lead to the failure of observing therapeutic effect continuously. Hereby, we proposed a

recombinant transbody against limitations of transbody built on the basis of natural antibody mentioned above.

In order to overcome the shortcomings of the transbody we constructed in our previous research, our novel recombinant transbody consisted of several gene elements: 1) For the recognition and binding between antigens and antbodies, complementarity-determining region (CDR) known as antigen-binding site of antibody, is selected as the active domain to minimum the molecular size of recombinant transbody while ensure the specificity and affinity between recombinant transbody and HBc(Stanfield, 2014). 2) Human Thioredoxin (hTRx) is used as a frame protein to stablilize the transbody under the reduction condition inside the cell, and hTRX has also been demonstrated to possess the ability to scavenge free radicals. In addition, as a frame protein to construct a gene fusion system, hTRX would effectively expose the active domain of our recombinant transbody(Anbanandam, Albarado, Tirziu, Simons, & Veeraraghavan, 2008; Borghouts, Kunz, Delis, & Groner, 2008). 3) TAT-PTD is preserved for transmembrane ability and contains a nucleus localization sequence which could lead recombinant transbody to nuclear(Vivès, Brodin, & Lebleu, 1997). 4) Homologue of N-terminus of the haemagglutinin of influenza viral envelope protein (HA20) is synthesized as endosome-releasing oligopeptide (EROP) to protect recombinant transbody from degradation by lysosome system of intracellular endocytosis(Ma et al., 2003). 5) His_6 and FLAG tag were fused for purification and identification of recombinant transbody.

Fortunately, we obtained a recombinant transbody named H2L2 with the above modification through prokaryotic expression system. Firstly, the optimal conformation of recombinant transbody had been designed and calculated by computer structural prediction software. We chose H2L2 as final active domain for recombinant transbody due to its affinity with the crystal structure of HBV capsid, and our recombinant transbody is named H2L2 after this active domain. The final structure of the recombinant transbody fused with all fuction parts (TAT-PTD, hTrx, CDR, HA20, 6*HIS and FLAG) had been further optimized for a correct folding to build a functional protein. We purified H2L2 using Ni^{2+}-NTA chromatography and ÄKTA protein purification system. After using nuclear magnetic resonance (NMR) verified H2L2 was completely folded by recording its hydrogen spectrum, we tested its transmembrane activity and ability of recognizing and binding to hepatitis B antigen. As a result, we successfully acquired an active recombinant transbody which

can effectively cross the membrane of hepatocytes and bind to hepatitis B antigen. These results indicated that we have taken a relatively successful first step towards an ideal therapeutic strategy against cccDNA of hepatitis B inside hepatocytes. The antiviral capacity of our recombinant antibody H2L2 needs to be further verified in our future studies to determine whether this recombinant antibody needs to be improved.

Results

Design and optimization the structure of recombinant transbody H2L2

We chose H2L2 as the final antigen binding domain for our recombinant transbody. First, the CDRs in the variable regions of heavy chain (VH) and light chain (VL) of HBV core antibody were screened. We randomly combined three CDRs on VH and VL with linker GSG (Fig.1A)(Hofacre et al., 2018), designing a total of 9 active domains, whose sequences are represented by HnLm (Table 1).

Table 1. Candidate amino acid sequences of active domain for recombinant antibody

HnLm	VHCDR-linker-VLCDR amino acids sequence
H1L1	TTSPGETVTLTCRS linker QSPWGETVTLTCRSS
H1L2	TTSPGETVTLTCRS linker PGVPARFSG
H1L3	TTSPGETVTLTCRS linker GAQTEDEAI
H2L1	WVQEKPDHLFTGLIGGTNNRAPG linker QSPWGETVTLTCRSS
H2L2	WVQEKPDHLFTGLIGGTNNRAPG linker PGVPARFSG
H2L3	WVQEKPDHLFTGLIGGTNNRAPG linker GAQTEDEAI
H3L1	GAQTEDEAIYFCALWTSNH linker QSPWQETVTCRSS
H3L2	GAQTEDEAIYFCALWTSNH linker PGVPARFSG
H3L3	GAQTEDEAIYFCALWTSNH linker GAQTEDEAI

Then, we applied several tools to simulate the structure of the active domain fusion with hTRx frame protein, which showed that hTRx could expose the active domain effectively (Fig.1B). The affinities between nine candidates' domains and antigens were predicted according to the crystal structure of HBV capsid. We found that the H1Lm protein had a stronger affinity in the H region ($P = 0.5268$), while HnL2 series proteins had a stronger affinity in the L region ($P = 0.5936$).

H1L1 and H1L2 were most likely to produce an immune response. Considering the negatively charged area on the spiral tip of the HBV capsid is the main response area between HBV capsid and its antigen, and only H2Lm contains a positive charged domain in its H region, the order of affinity strength is: H2L2> H2L1>H2L3. Therefore, we chose H2L2 as the final antigen binding domain for recombinant transbody, which is named H2L2 after this active domain (Fig.1C).

The location of each gene element was then optimized as correct folding is the fundamental for a functional protein (Fig.2D). The simulation structural model of H2L2 showed that the active domain of the recombinant transbody can be exposed properly (Fig.2A). The surface of the H2L2 is basically electrically neutral except the TAT region, and the neutral charge distribution may contribute to protein stability in solution or in cells (Fig.2B). TAT-PTD domain at the N-terminal of H2L2 is positively charged, providing a basis for recombinant transbody to pass through the cell membrane and enter hepatocytes (Fig.2C). Compelete amino acids sequence of H2L2 recombinant transbody is shown in supplementary data (Sup.1).

Successfully expression and purification of H2L2 recombinant transbody

Recombinant transbody H2L2 was successfully expressed and purified through prokaryotic expression system. The target gene was amplified from plasmid pUC57-H2L2. PCR product was purified and cloned into pET-28a vector to construct recombinant expression plasmid pET-28a-SUMO-H2L2, and the plasmid had fused with SUMO tag which would help our recombinant transbody to fold properly (Fig.3A). Nucleotides sequencing further confirmed the successful construction of pET-28a-SUMO-H2L2 recombinant plasmid (Sup.2). The soluble recombinant transbody H2L2 was initially purified by Ni^{2+}-NTA chromatography (Fig.3B). The expression of the H2L2 recombinant transbody is further verified by Western Blot using an anti-His_6 antibody as the H2L2 recombinant transbody contains a His_6-tag on its N-terminal (data not shown).

Recombinant transbody H2L2 was successfully purified by ÄKTA protein purification system and NMR results showed that H2L2 had fold completely (Fig.4). H2L2 recombinant transbody was purified by ÄKTA protein purification system, which purified proteins by thedatair size of molecular weight. The molecular weight of protein that appeared at 45 minutes was about 45 kDa, consistent with the size of recombinant transbody H2L2 at 37kDa (Fig.4A). After removing the

fusion tag SUMO from the recombinant transbody H2L2 using ULP1 protease, hydrogen spectrum was recorded by nuclear magnetic resonance (NMR) indicating recombinant transbody H2L2 was completed folded. For unfolded proteins, the water molecules outside the protein are in the same environment; thus, hydrogen spectrum summit is concentrated on X-axis 4.7 (the water peak) (Sup.2). For folded proteins, due to the significant difference in the environment of water molecules surrounding the folded protein, the hydrogen spectrum summit broadened. So hydrogen spectrum peaks appeared at the position less than the x-axis 0.5, showing that H2L2 is completely folded (Fig.4B). In the end, we obtained 5mg/ml recombinant transbody H2L2 and stored at -80℃.

Recombinant transbody H2L2 had transmembrane ability

Immunofluorescence staining results indicated that recombinant transbody H2L2 can effectively cross the hepatocytes' membrane, and with/without SUMO tag removed (group H/S) H2L2 can both cross the membrane, but the efficiency of group H was significantly higher than group S. To our surprise, recombinant transbody H2L2 with SUMO tag removed (group H) tended to accumulate around the nucleus inside hepatocytes (Fig.5). DAPI (blue fluorescence) and phalloidin (green fluorescence) showed clear outlines of the nucleus and cells of Hep3B cells. Red fluorescence was evident inside cells of H2L2 group, indicating that our recombinant transbody can effectively cross the cell membrane. In order to find out whether SUMO tag would influence the transmembrane ability, we incubated H2L2 with/without SUMO tag removed (group S/H) with Hep3B cells respectively. It turned out that the intensity of red fluorescence in group S was significantly lower than group H, indicating SUMO tag may disturb the transmembrane ability of recombinant transbody (Fig.5). Besides, different amounts of H2L2 (5.0 μg/μL, 16 μL/64 μL) were incubated with Hep3B cells respectively, to find out if the amount of H2L2 would influence the transmembrane efficient of H2L2. We found that compared with the control group (PBS) , H2L2 can basically all cross the cell membrane, but the fluorescence signal was weaker when the amount was small. Incubating time showed no influence on transmembrane effectivity, and H2L2 could effectively cross the cell membrane within 4 hours (Fig.5).

Recombinant transbody H2L2 can effectively bind to Hbc antigen

Immunohistochemistry (IHC) and the analysis of Integrated Optical Density (IOD) showed that recombinant transbody H2L2 can effectively bind to HBcAg within HBV infected cells (Fig.6).

HBcAg+ patients' liver tissues were stained brown in postive control (HBcAb), SUMO-H2L2 and H2L2 group while negative group (protein elution buffer) was not stained brown, and none of HBcAg- patients' liver tissues were stained brown (Fig.6A). IOD was calculated by ImageJ (Table 2). The differences of IOD between HBcAg+/ HBcAg- patients and within four groups were statistically significant ($P < 0.01$) (Fig.6B). Interestingly, different from transmembrane activity, SUMO tag seems had no influence on antigen binding ability of recombinant transbody H2L2.

Table 2 IOD of immunohistochemical experiments

IOD	Negative control	Positive control	SUMO-H2L2	H2L2
Average IOD of group HBcAg+	1546.525	23566.982	27179.533	27646.957
SD IOD of group HBcAg+	25.546	132.515	522.133	98.936
Average IOD of group HBcAg-	225.344	244.276	218.814	194.897
SD IOD of group HBcAg-	11.470	8.200	28.582	5.296

Materials and methods

Design and optimization of recombinant transbody structure

Computer structural calculation software (I-TASSER, SWISSMODEL, JPRED, PREPSITE 2, HADDOCK, RAPTOR-X, PHYRE 2) was used for designing and optimizing the recombinant transbody structure, and the CDR domain of heavy chain and light chain variable region of HBc antibody was screened. The selected CDR domains were fused with hTrx frame protein contributing to the active domain of our recombinant transbody. The affinity between different candidate active domains and HBcAg was predicted according to the hydrophilic, hydrophobic and electrical properties of recombinant transbody. The three-dimensional structure of the recombinant antibody was obtained through protein homology modelling, and the localization of different gene elements were optimized to predict the model of the functional domain after protein folding to obtain the optimized structure of the recombinant transbody.

Bacterial strains and plasmid

H2L2 cDNA was amplified from synthetic plasmid pUC57-H2L2 synthesized by BGI (Beijing, China) using primers (5′-GAACAGATTGGTGGTATGCACCACCACCACCACCACTAC-3′) and (5′-GTGGTGCTCGAGCTATCATTTGTCGTCGTCGTCTTTGTAGTC-3′). PCR product was purified and cloned into pET-28a vector using Gibson Assembly method to construct recombinant expression plasmid pET-28a-SUMO-H2L2. The resulting plasmid pET-SUMO-H2L2 was transformed into *E. coli* DH5α cells. At least five independent clones of each plasmid were sequenced.

Expression and purification assay

The resulting pET-28a-SUMO-H2L2 recombinant plasmid was transformed into BL21(DE3) cell and then was induced by 1mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and harvested after overnight incubation at 18 ℃ in 1L LB medium. The culture was collected by centrifugation at 5422*g for 10minutes at 4 ℃, and the cell pellet was resuspended in 30 mL binding buffer (250 mM NaCl, 50 mM NaH2PO4, 2 mM TCEP, pH 8.0) and lysed by sonication. Lysate was then centrifuged at 47,850 *g for 30 minutes at 4℃. The soluble H2L2 recombinant transbody in the supernatant of the cell lysate was initially purified by Ni^{2+}–NTA chromatography (GE Healthcare Life Sciences). The supernatant was added to the column packed with Ni2+-NTA His-binding resin and washed with wash buffer (300 mM NaCl, 50 mM NaH2PO4, 20 mM Imidazole, pH 8.0), and then eluted by elution buffer (300 mM NaCl, 50 mM NaH2PO4, 250 mM Imidazole, pH 8.0). The presence of the protein in the fractions was monitored using the Bradford assay, 20% SDS-PAGE and absorbance at 280 nm on the Nanodrop. The SUMO tag was removed using SUMO protease Ulp1 and passed through His-Trap column twice. The cleaved protein was dialysed with the storage buffer (250 mM NaCl, 50 mM KH2PO4, 2 mM TCEP, pH 7.0) at 4 °C overnight and concentrated by ultrafiltration with a 10 kDa cut-off filter for subsequent experiments. 5 mg/mL H2L2 protein was collected and stored at -80 °C. H2L2 recombinant transbody was further purified by ÄKTA protein purification system (General Electric Company, Boston, USA).

SDS-PAGE electrophoresis

12 μL protein sample was mixed with 3 μL 5×loading buffer and then heated to 95°C for 5 minutes. 10 μL of mixtures were loaded in each lane of 20% polyacrylamide gels in the Mini Trans-Blot

Cell system (BIO-RAD) running at 210 V for 1 h. Gels were stained with Coomassie brilliant blue for no less than 10 minutes followed by destaining in water overnight.

Western Blot analysis

The elution of IPTG induced/none-induced H2L2 protein were resolved by SDS–PAGE using a 12% gel. The resolved proteins were transferred to a nitrocellulose membrane, and western blot analysis was performed as proposed previously. The resulting western blot signals were visualized using a colorimetric detection kit, according to the manufacturer's instructions.

Nuclear Magnetic Resonances (NMR) assay

Size exclusion chromatography purified H2L2 proteins were concentrated to at least 0.1 mM for 1D analysis. All NMR experiments were conducted on a Bruker 600 MHz (Avance III) at 298 K with 128 scans. The sample was in a buffer containing 250 mM NaCl, 50 mM KH2PO4, 2 mM TCEP, pH 7.0. The water resonance was presaturated during the relaxation delay and the chemical shift of the signal peak was determined with referencing to D2O (4.72 ppm). The spectra was acquired using Topspin 4.1.4, and processed and analyzed in NMRPipe. The phase and baseline of the spectra were corrected manually.

Cell lines and Immunofluorescence staining assay

The human HBV-infected cell line Hep3B (EK-bioscience, Shanghai, China) were obtained and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 mg/mL strepto-mycin, and 2 mmol/L L-glutamine at 37 °C in an atmosphere of 5% CO_2 and a 100% humidity. Hep3B cells were grown on glass coverslips to about 70% confluency. After the culture medium was discarded, 16μL/64μL of 0.5μg/mL recombinant transbody H2L2 dissolved in PBS were incubated with Hep3B for 4h/8h respectively, meanwhile 16μL/64μL of PBS were added to the cells as control group. After washed three times with PBS, the cells were fixed with 4% paraformaldehyde, then washed three times with PBS, permeabilized with 0.05 mL of 0.1% Triton X-100 and blocked with 0.05 mL of 20% bovine serum/PBS. 100 μL of rabbit anti-His_6 (1:200) was added to the cells, after incubating overnight at 4°C, washed three times with PBS. 100 μL goat anti-rabbit IgG (1:200) was added to the cells, while 100 μL of phalloidin (1:1000) was added to the cells and incubating away from light for 2 hours at room

temperature, washed three times with PBS. 100 μL of DAPI (blue) was added to the cells incubating away from light for 20 minutes at room temperature. The cells were washed again and then examined by CKX31-A11PHP fluorescence microscopy. Silicon Graphics workstation DP-2-BSW was used to capture the cells.

Immunohistochemistry (IHC) assay

Liver tissues fixed in formalin were dehydrated, embedded in paraffin, and sectioned to a thickness of 3 μm; the sections were deparaffinized and rehydrated. After blocking endogenous peroxidases with 0.3% H_2O_2 for 10 minutes, non-specific binding sites were blocked with 10% goat serum for 10 minutes. After washing three times with PBS, the sections were incubated with 100 μL 5.0 mg/mL of H2L2-SUMO/H2L2 recombinant transbody dissolved in PBS overnight at room temperature, while 100 μL of 200-fold-diluted rabbit anti-human HBcAb and 100 μL PBS as control group were added to the tissue. Washed three times with PBS, added 100 μL of 200-fold-diluted rabbit anti-His_6 incubating for 50 minutes. Washed three times with PBS, then add 100 μL of 200-fold-diluted goat anti-rabbit IgG incubating for 30 minutes. Immunostaining was developed using 3,3'-diaminobenzidine (DAB), and sections were counterstained with haematoxylin. The Integrated Optical Density were calculated with ImageJ.

Statistical analysis

The results were presented as the mean ± standard deviation (SD). The statistical significance of the differences was evaluated by t-test in SPSS. For all comparisons, differences with $P < 0.05$ (**), $P < 0.01$(**), and $P < 0.001$(***) were considered statistically significant.

Disccusion

Consensus had been reached that the persistence of cccDNA in infected hepatocytes is the key obstacle for CHB therapies. Years of devoted research aims at potential therapeutic strategies targeting cccDNA. To date, there are two main classes of strategies: indirect strategies and direct strategies. However, few of them had entered the clinical research due to their limitations. In view of these problems, we constructed a transmembrane antibody targeting HBc inside hepatocytes in our previous study and confirmed the antiviral ability of the transbody. Even so, there are unignorable limitations of the previous transbody as mentioned in the introduction. Thus,

optimization of this transbody is necessary for further exploration of an effective treatment potion for HBV infection.

In this study, we designed a recombinant transbody fused with several fuctional elements against limitations that appeared in the previous study. In other research, single-domain antibodies (VHHs) and single-chain antibody fragments (scFv) were used as the active domain for binding HBc(Serruys, Van Houtte, Farhoudi-Moghadam, Leroux-Roels, & Vanlandschoot, 2010; Serruys, Van Houtte, Verbrugghe, Leroux-Roels, & Vanlandschoot, 2009). However, their stability and affinity are not ideal as an antibody. Therefore, we chose CDR as the active domain to a minimum molecular size of our recombinant transbody while ensured the specificity and affinity between recombinant transbody and HBc. Candidate active domains were selected according to the predicted affinity between the active domain and HBV capsid, and H2L2 may be the idealist active domain due to this predication, therefore our recombinant transbody was named after H2L2. hTRX was chosen to be the frame protein for recombinant which had a special advantage as a human protein that will cause no immunogenicity. According to the prediction structure of recombinant transbody, hTRX could fully expose the active domain H2L2. The structural simulation was the cornerstone of constructing recombinant protein and the subsequent expression. We further optimized the insert location of each functional element to ensure their function could be properly performed. However, the actual folding state of recombinant transbody H2L2 may not conform to our prediction, functions of the recombinant transbody H2L2 needed to be further verified.

E.coli is widely used in protein expression due to its advantages: short culture cycle, low production cost, simple operation and genetic stability(Khow & Suntrarachun, 2012). Nevertheless, *E. coli* lacks endoplasmic reticulum, and molecular chaperones that help proteins fold in eukaryotes are the main limitations of prokaryotic express systems(de Marco, 2009). To obtain the maximum amount of right folded recombinant transbody, SUMO is selected as a fusion protein tag assisting with soluble protein expression(H. Wang et al., 2010). Gibson assembly was used to replace the double enzyme digestion method to construct the pET-SUMO-H2L2 recombinant plasmid, to ensure that the recombinant plasmid would not introduce any redundant bases to affect the protein expression during the construction process and the operation is simple. Recombinant transbody H2L2 was expressed and purified successfully.

NMR assay showed recombinant transbody H2L2 was completely folded, the function of the protein is further verified by immunofluorescence and immunohistochemistry assay. Results

showed H2L2 recombinant transbody could effectively cross the cell membrane and group H2L2 with SUMO tag removed tended to accumulate around the nucleus due to the nucleus signal sequence carried in TAT PTD. Recombinant transbody also has the ability to bind HBcAg in HBcAg+ patients. To our surprise, these results demonstrated that our recombinant transbody already had a basic ability for antiviral activity inside hepatocytes. This recombinant transbody was an encouraging exploration of therapeutic strategies against hepatitis B infection. The antiviral activity of the recombinant antibody will be further verified in subsequent studies.

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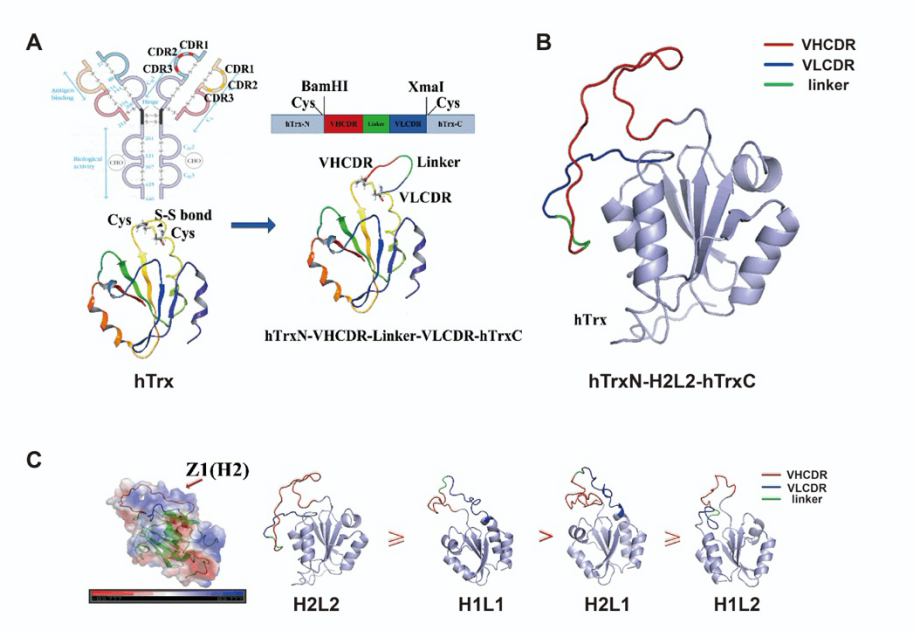

Fig 1. Design and optimization the antigen binding domain of recombinant transbody. (A) A simulation map of the HnLm domain of recombinant antibody; (B) Structure prediction of antigen binding domain H2L2 fused with hTRx frame protein; (C) Surface charge distribution of antigen binding domain HnLm fused with hTRx frame protein and the affinity prediction between candidate domains.

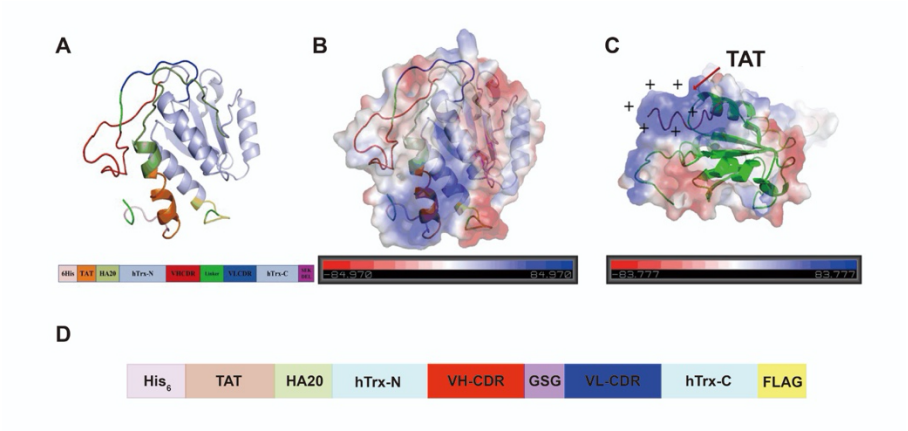

Fig 2. Structure prediction and optimization of insert location of fuctional elements within recombinant transbody. (A) Structure prediction of recombinant transbody H2L2 with fused fuctional elements; (B) Surface charge distribution of recombinant transbody H2L2; (C) TAT domain charging state in recombinant transbody H2L2; (D) Final insert location of functional elements in recombinant transbody H2L2.

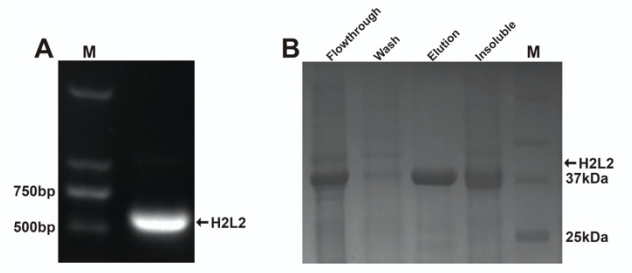

Fig 3. Expression of recombinant transbody H2L2. (A) Gel electrophoresis of target gene amplified by PCR reaction. M: DL2000 DNA Marker; arrow: target gene H2L2; (B) SDS-PAGE electrophoresis of H2L2 recombinant transbody induced in 1L LB medium. M: marker; arrow: target protein H2L2.

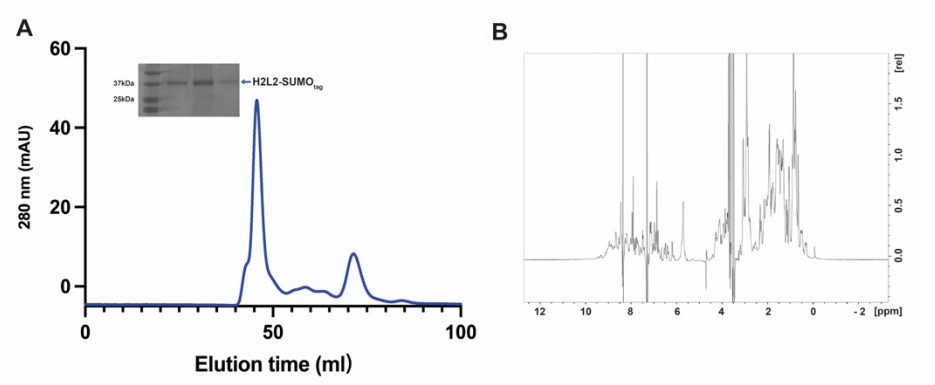

Fig 4. Purification and NMR experiment of recombinant transbody H2L2. (A) Purification of H2L2 recombinant transbody using ÄKTA protein purification system. Blue arrow: target protein H2L2; (B) Hydrogen spectrum recorded by NMR of completely folded recombinant antibody H2L2.

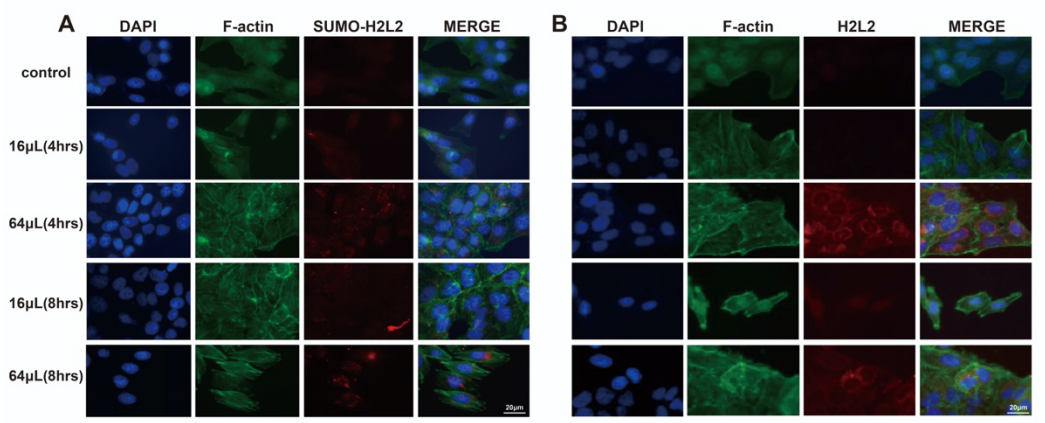

Fig 5. Recombinant transbody H2L2 had transmembrane ability. (A) Immunofluorescence results of group S (recombinant transbody H2L2 with SUMO tag). DAPI: blue; F-actin: green; SUMO-H2L2/H2L2: red; 16/64μL: amount of target protein H2L2 incubate with Hep3B cells; 4/8hrs: times of target protein H2L2 incubate with Hep3B cells; (B) Immunofluorescence results of group H (recombinant transbody H2L2 with SUMO tag removed). DAPI: blue; F-actin: green; SUMO-H2L2/H2L2: red; 16/64μL: amount of target protein H2L2 incubate with Hep3B cells; 4/8hrs: times of target protein H2L2 incubate with Hep3B cells.

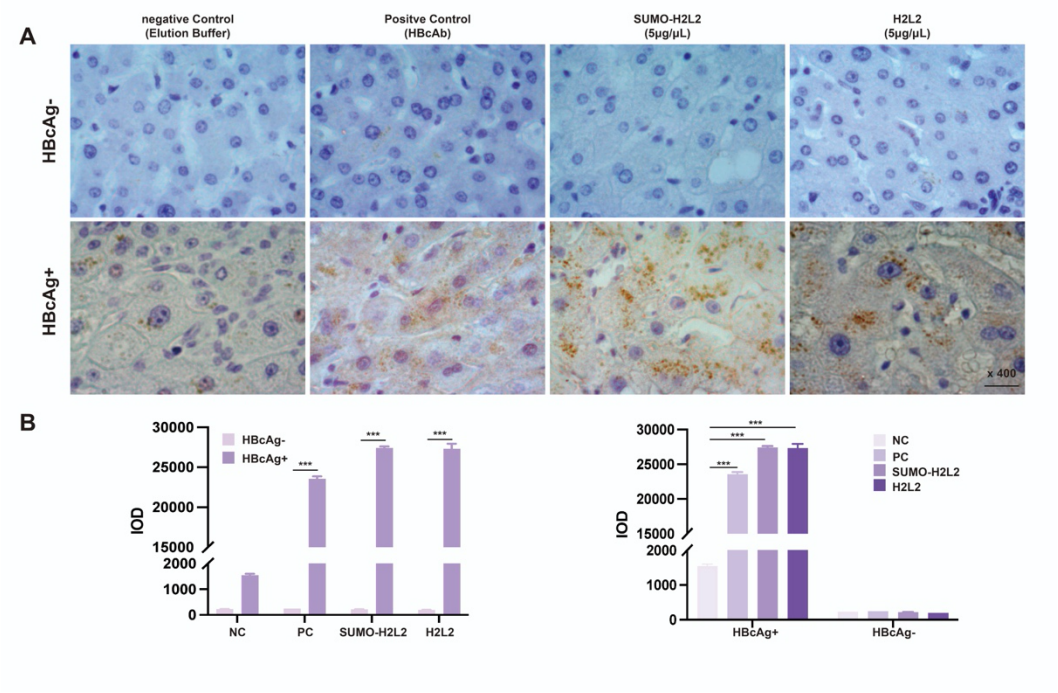

Fig 6. Recombinant transbody H2L2 can effectively bind to Hbc antigen in liver tissue. (A) Immunohistochemistry results of H2L2 recombinant transbody binding HBcAg. Positive control: commercial HBcAb; Negative control: elution buffer; SUMO-H2L2: H2L2 recombinant transbody with SUMO tag; H2L2: H2L2 recombinant transbody with SUMO tag removed; (B) Left: statistic histogram of HBcAg+ patients comparing HBcAg- patients in each experimental group. PC (positive control): commercial HBcAb; NC (negative control): elution buffer; SUMO-H2L2: H2L2 recombinant transbody with SUMO tag; H2L2: H2L2 recombinant transbody with SUMO tag removed *** $P < 0.001$; Right: Statistic histogram of each experimental group comparing in HBcAg+ patients and HBcAg- patients. PC (positive control): commercial HBcAb; NC (negative control): elution buffer; SUMO-H2L2: H2L2 recombinant transbody with SUMO tag; H2L2: H2L2 recombinant transbody with SUMO tag removed *** $P < 0.001$.